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RhoA activity is required for fibronectin assembly and counteracts β1B integrin inhibitory effect in FRT epithelial cells

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SUMMARY

FRT thyroid epithelial cells synthesize fibronectin and organize a network of fibronectin fibrils at the basal surface of the cells. Fibronectin fibril formation is enhanced by the overexpression of the ubiquitous β1A integrin and is inhibited by the expression of the dominant-negative β1B subunit. We tested the hypotheses that RhoA activity might mediate the integrin-dependent fibronectin fibrillogenesis and might counteract β1B integrin inhibitory effect. FRT-β1A cells were transfected with a vector carrying a dominant negative form of RhoA (RhoAN19) or treated with the C3 transferase exoenzyme. Both treatments inhibited fibronectin assembly and caused loss of actin microfilaments and adhesion plaques. On the other hand, FRT-β1B cells were transfected with the constitutively activated form of RhoA (RhoAV14) or treated with the *E. coli* cytotoxic necrotizing factor 1, which directly activates RhoA. Either treatment restored microfilament and adhesion plaque assembly and promoted fibronectin fibril organization. A great increase in fibronectin fibril assembly was also obtained by treatment of FRT-β1B cells with TGF-β.

Our data indicate that RhoA is required to promote fibronectin matrix assembly in FRT cells and that the activation of the signal transduction pathway downstream of RhoA can overcome the inhibitory effect of β1B integrin.

Key words: Integrin, Fibronectin, Epithelial cell, RhoA, TGF-β

INTRODUCTION

Fibronectin (FN) matrix assembly is a dynamic process, in which soluble dimeric FN molecules are assembled into an insoluble, disulfide-bond stabilized, fibrillar matrix (Aguirre et al., 1994; Chernousov et al., 1991; Ichihara-Tanaka et al., 1995; Morla and Ruoslahti, 1992; Mosher et al., 1991, 1992; Schwarzbauer, 1991; Sottile and Wiley, 1994). Several insights into the mechanism of formation of FN fibrils have recently been provided. It has been found that the expression on the plasma membrane of specific FN binding integrins (Dzamba et al., 1994; Wu et al., 1993) is required. Among those, α5β1, αvβ3 and αIIbβ3 integrins have been identified (Giancotti and Ruoslahti, 1990; Wennerberg et al., 1996; Wu et al., 1993, 1995). The activation state of integrins, i.e. the acquisition of a high affinity state for FN, is needed for fibrillogenesis in CHO cells (Wu et al., 1995). However, post-occupancy events following integrin activation and involving the β subunit cytoplasmic domain and an intact cytoskeleton are also required (Faull et al., 1993; Wu et al., 1995). Newly assembled FN fibrils coalign with bundles of actin filaments and with focal adhesions (Heggeness et al., 1978; Hynes and Destree, 1978; Singer and Paradiso, 1981; Wu et al., 1995), suggesting that actin stress fiber, adhesion plaque and FN fibril formation are coordinately regulated (Ali and Hynes, 1977; Christopher et al., 1997; Sechler and Schwarzbauer, 1997).

Upon interaction with the substrate the integrins cluster and associate with a variety of cytoplasmic proteins to form focal complexes. Integrin-mediated adhesion induces cytoskeletal organization, leading to actin stress fiber formation (Burridge et al., 1988). Binding of integrins to their extracellular matrix also results in the transduction of tyrosine kinase-mediated signals (Clark and Brugge, 1995; Parsons, 1996; Schwartz et al., 1995). Several observations indicate the requirement of the small GTP binding protein RhoA in integrin signaling (Defilippi et al., 1997; Parsons, 1996; Schwartz et al., 1996). It is known that RhoA activation is required for the assembly of microfilament bundles and of adhesion plaques (Ridley and Hall, 1992), such as that induced by growth factors and by LPA (Ridley, 1994). However, the formation of stress fibers and focal adhesions can be obtained in the absence of added growth factors by plating cells on extracellular matrix proteins or by addition of the GRGDS peptide, which is recognized by several integrins (Barry et al., 1997). It has been demonstrated that microinjecting the cells with the RhoA inhibitor C3 transferase exoenzyme can block these effects (Barry et al., 1997), indicating that integrin-mediated actin cytoskeleton assembly may require RhoA activation. LPA has also been shown to

promote FN matrix assembly (Zhang et al., 1994). This effect appears to be correlated to actin stress fiber formation, cell contraction and increased FN binding (Zhang et al., 1997).

We recently demonstrated that β1 integrins are involved in the control of FN matrix assembly in FRT thyroid epithelial cells (Cali et al., 1998). In subconfluent cultures the FN matrix is almost exclusively organized at the basal cell surface. Stable expression of the human β1A integrin in FRT cells (FRT-β1A cells) causes an increase in FN assembly that is paralleled by an increase of microfilaments and adhesion plaques formation. Expression of the β1B (Altruda et al., 1990) dominant-negative variant (FRT-β1B cells) determines their inability to assemble FN fibers with concomitant loss of actin filaments and adhesion plaques (Cali et al., 1998).

The aim of this study was to investigate the role of RhoA in promoting FN matrix organization. We inhibited RhoA function in FRT-β1A cells, either by expressing a dominant-negative form of RhoA (RhoAN19) or by treating cells with the C3-transferase exoenzyme (Chardin et al., 1989; Nemoto et al., 1991), and we observed a dramatic reduction of FN assembly. The ability to organize FN fibrils was instead restored in FRT-β1B cells by transfecting a constitutively activated form of RhoA (RhoAV14), or by treatment with the cytotoxic necrotizing factor (CNF1) (Fiorentini et al., 1997). We further demonstrated that treatment with TGF-β counteracted the dominant-negative effect of the β1B integrin subunit.

These results indicate that RhoA has a role in the integrin-mediated FN assembly in epithelial cells and suggest a possible mechanism by which β1B interferes with this process.

MATERIALS AND METHODS

Antibodies and reagents

Rabbit antiserum to rat FN was from Chemicon International (Temecula, CA); anti-paxillin mAb was from Zimed Lab. Inc. (San Francisco, CA); anti-phosphotyrosine (PT 66) mAb was from Sigma Chemical Co. (St Louis, MO); anti-c-myc (9E10) and anti-RhoA mAbs were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); mAb against α5β1 (PB1) was a gift of R. L. Juliano (University of North Carolina-Chapel Hill, Chapel Hill, NC; Brown and Juliano, 1985); horse radish peroxidase-conjugated anti-mouse antibody was from Amersham (Amersham, Buckinghamshire, UK); fluoresceinated avidin was from Oncor (Galthensburg, MD). CNF1 (cytotoxic necrotizing factor 1) was a kind gift of C. Fiorentini (Istituto Superiore di Sanità, Roma, Italy). It was purified from *E. coli* BM2 strain as previously described (Falzano et al., 1993). TGF-β, type 1, was from R & D System Inc., (Minneapolis, USA). C3 ADP-ribosyltrasferase was from Sigma. It was dissolved at a concentration of 100 μg/ml in 120 mM KCl, 10 mM Tris/HCl, pH 7.4.

Cell cultures

FRT cells and stable clones of FRT-β1A and FRT-β1B cells were cultured in Falcon tissue culture plastic dishes (Becton Dickinson Labware, Lincoln Park, NJ) in Coon's modified Ham's F12 medium (Sigma) containing 5% FBS (Gibco, Paisley, UK), penicillin, streptomycin and 500 μg/ml of G418 (Sigma). For cell transfections and for all immunofluorescence assays 1.5×10^5 cells were plated onto 12 mm diameter glass coverslips.

Constructs and transfections

pEXVmyctag V14Rho was kindly provided by A. Hall (MRC, University College of London); RhoAN19 was a gift of R. A. Cerione (Cornell University, Ithaca, New York).

The pEFmyctag RhoAN19 was generated by PCR amplification of the RhoAN19 coding region from the original plasmid (provided by A. Cerione) with a 5′ primer and a 3′ primer containing at their ends the *Bam*HI and *Eco*RI sites, respectively. The fragment was then subcloned in the corresponding sites of the expression vector EFplink that was previously modified with the addition of a myc tag by R. Treisman. The construct was sequenced to control the fidelity of the frame between the myc tag and the inserted fragment. The DNA of all the plasmids was prepared by Qiagen cartridges (QIAGEN GmbH, Germany) and used for cell transfections.

FRT, FRT-β1A or FRT-β1B cells were transfected directly on 12 mm diameter glass coverslips with 0.5-2 μg of pEXVmyctag V14Rho or pEXVmyctag RhoAN19 plasmid and 1.25-5 μg of Lipofectin (Gibco BRL Life Technologies Paisley, UK). Transfections were performed as suggested by the factory with minor modifications. Briefly, cells were seeded onto 12 mm glass coverslips in a 24-well cell culture cluster (Costar, Cambridge, MA) in regular medium and incubated overnight. The culture medium was replaced with OPTIMEM 1 (Gibco BRL) 3 hours before transfection. The DNA/liposomes ratio was as suggested (1:2.5) and the final volume on the coverslips was 200 μl.

Immunofluorescence

Subconfluent cells on glass coverslips were fixed for 20 minutes with 4% paraformaldehyde (Sigma) in PBS containing 0.9 mM calcium and 0.5 mM magnesium (PBS CM) at room temperature, washed twice in 50 mM NH_4Cl in PBS CM and twice in PBS CM. Cells were permeabilized for 5 minutes in 0.5% Triton-X 100 (Bio-Rad) in PBS CM and washed twice, for 10 minutes, in 0.2% gelatin (Sigma) in PBS CM.

Cells were then incubated for 1 hour with the primary antibodies diluted in 0.5% BSA (Sigma) in PBS. After three washes with 0.2% gelatin in PBS CM cells were incubated for 20 minutes with the appropriate rhodamine- or fluorescein-tagged goat anti-mouse or anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA), diluted 1:50 in 0.5% BSA in PBS. To visualize actin filaments, permeabilized cells were incubated with a 1:70 dilution of rhodamine-conjugated phalloidin (Sigma) for 20 minutes. After final washes with PBS, the coverslips were mounted on a microscope slide using a 50% solution of glycerol in PBS and examined with a Zeiss Axiophot microscope.

Samples were observed by three investigators independently, without knowledge of the experimental conditions. At least 100 fluorescence-positive and negative cells were counted for each experimental condition and representative fields were photographed using Ektachrome P1600 colour reversal film. Images were also acquired with a CCD camera (Sensys, Photometrics, Tucson, AZ). In either case the exposure length was fixed using the control as the index.

Western blot

FRT and FRT-β1B cells were seeded at low confluence onto 100 mm diameter plastic dishes (Falcon). After 1 day the cells were washed three times, incubated overnight in medium without serum and then treated for 3 hours with 0.2 ng/ml CNF1. Cells were washed twice with PBS and lysed for 20 minutes in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8, 5 mM EDTA, pH 8, 10 mM NaF, 10 mM $Na_4P_2O_7$, 0.4 mM Na_3VO_4, 1% Nonidet NP-40) containing protease inhibitors. Cells were then scraped and centrifuged for 10 minutes at 4°C at 14,000 rpm (21,000 ***g***). The pellets were discarded and protein concentration was determined using the Bio-rad protein assay (Bio-rad). 40 μg of protein were solubilized in Laemmli sample buffer, boiled for 5 minutes, and analyzed by SDS/6-15%PAGE.

Gels were blotted onto nitrocellulose filters (Amersham) in a Bio-Rad apparatus (Bio-Rad). The filters were washed extensively with TTBS (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.05% Tween 20), blocked at room temperature for 2 hours with 1% non-fat dry milk

(NFDM; Bio-Rad) in TBS (150 mM NaCl, 20 mM Tris-HCl, pH 7.5), washed twice with TTBS and once with TBS, and incubated for 1 hour at room temperature with the anti-RhoA mAb (Santa Cruz) diluted 1:1000 in TBS containing 0.5% NFDM. Subsequently, the filters were washed extensively with TTBS and with TBS and incubated for 1 hour at room temperature with horse radish peroxidase-conjugated anti-mouse antibody (Amersham) diluted 1:1000 in 0.5% TBS. After extensive washing with TTBS and with TBS, the filters were developed using an ECL detection method (Amersham), according to the manufacturer's directions.

Biotinylation of FN

80 μg (80 μl of 1 mg/ml) bovine serum FN (Sigma) was allowed to react with 400 μg (2 μl) of NHS-LC biotin (Pierce, Rockford, IL) in 420 μl PBS CM, for 20 minutes, at 4°C. The solution was dialyzed against PBS CM for 24 hours at 4°C. 1.5×10^5 FRT-β1A cells were seeded onto 12 mm diameter glass coverslips and treated with 4 μg (125 μl) of biotinylated FN. Immunofluorescence was performed as described. Biotinylated FN was detected with fluorescein-tagged avidin (Oncor, Galthensburg, MD).

RESULTS

FRT-β1A cells organize an extracellular matrix containing both the cell-secreted FN and the serum FN

Subconfluent FRT cells cultured in the presence of 5% foetal calf serum were shown to assemble FN fibrils at the basal surface of the cells (Cali et al., 1998). In most of the experiments shown in this paper we have used two FRT-derived cell lines, FRT-β1A and FRT-β1B, which express the human β1A and β1B integrin subunit, respectively (Cali et al., 1998). FRT-β1A cells were able to deposit a much more abundant FN matrix (Fig. 1a) compared to wild-type FRT cells. In serum-free medium, a reduced amount of extracellular FN was still assembled (Fig. 1b), indicating that the cells can organize into fibrils the FN molecules that they synthesize and secrete. FRT-β1B cells, on the contrary, did not assemble FN fibrils in significant amount either in the presence or in the absence of serum (Fig. 1c-d). To demonstrate that FRT-β1A cells were indeed able to organize the FN present in the medium into fibrils, subconfluent cultures were grown in serum-free conditions in the presence of soluble biotinylated FN. The FN matrix deposited was detected by an anti-rat FN antibody (Fig. 1e) while the presence of biotinylated FN in the insoluble matrix was revealed by rhodamine-conjugated streptavidin (Fig. 1f). FN fibrils were labeled with fluorescent markers (Fig. 1e-f), indicating that newly synthesized as well as exogenous FN molecules participate in the assembly of the same extracellular FN matrix.

α5β1 integrin is involved in the process of FN assembly

The involvement of the α5β1 integrin dimer in the fibrillogenesis process has been tested by interfering with the endogenous α5β1 activity. This was achieved by including in the culture medium the blocking antibody PB1 (Brown and Juliano, 1985; Wu et al., 1995) that is directed against the α5β1 FN receptor. FRT-β1A cells were cultured for 18 hours in the presence of PB1 and then analyzed by immunofluorescence for the presence of assembled FN. A great reduction in the amount of cell-associated fibrils was seen in PB1-treated cells (Fig. 1g) as compared to control cells cultured in regular medium (Fig. 1h) or in the presence of non-specific mAbs (data not shown). This result indicates that α5β1 is the main integrin involved in the process of FN fibril organization in FRT cells.

The expression of RhoAN19 inhibits FN matrix assembly

We have previously reported that in FRT-β1A cells the FN fibrillar network is paralleled by the organization of a well developed actin cytoskeleton and by the formation of a great number of adhesion plaques (Cali et al., 1998). To assess the role of RhoA in the process of extracellular FN deposition we investigated the effect of the expression of the dominant-negative form of RhoA, RhoAN19, in FRT-β1A cells. Cells were transiently transfected with an expression vector carrying the dominant-negative form of RhoA fused to a myc tag. After 24 hours cells were fixed and double-stained for immunofluorescence detection with the anti-myc antibody (Fig. 2a,c,e) and with rhodamine-conjugated phalloidin (Fig. 2b), anti-paxillin antibody (Fig. 2d) or anti-FN antibody (Fig. 2f). A significant reduction in microfilament and adhesion plaque assembly, and FN matrix organization, was found. It was observed that 73±13% of RhoAN19-expressing cells had reduced amounts of actin filaments and of FN matrix with respect to 29±9% of mock transfected cells. These data indicate that RhoA activity is required not only to assemble microfilaments and adhesion plaques but also to organize FN fibrils.

Inactivation of endogenous RhoA by C3 transferase exoenzyme inhibits FN fibril formation

C3 ADP-ribosyltransferase of *C. botulinum* is a useful tool for investigating Rho-protein-mediated cellular responses because it specifically inactivates Rho protein (Aktories et al., 1992; Quilliam et al., 1989; Sekine et al., 1989). Although in other experimental systems the C3 transferase exoenzyme has been shown to penetrate the cells with some difficulty, this was not the case with FRT cells, which appeared to be quite sensitive to the action of the toxin. 1.5 μg/ml C3 transferase was added to FRT-β1A cells that had been cultured for 48 hours in standard conditions. After 8 or 18 hours cells were fixed and double-stained with rhodamine conjugated-phalloidin and with the anti-FN antibody (Fig. 3). After 8 hours of C3 transferase we observed that some cells showed a loss of microfilaments and a reduction in FN fibrils. These cells were frequently in small clusters (Fig. 3a-b) that were easily detected within the culture. By 18 hours of treatment most of the cells were found to be without microfilaments (Fig. 3c) and only residual deposits of FN, mostly in the form of irregular cables, were present (Fig. 3d). A similar result was obtained when wild-type FRT cells were treated with C3 transferase (data not shown). It appears that C3 inhibition of Rho activity leads to progressive disruption of the actin cytoskeleton, inhibition of organization of FN fibrils, and also removal of previously organized FN matrix.

The expression of RhoAV14 in FRT-β1B cells restores the ability to assemble the FN matrix

FRT-β1B cells do not organize actin filaments and adhesion

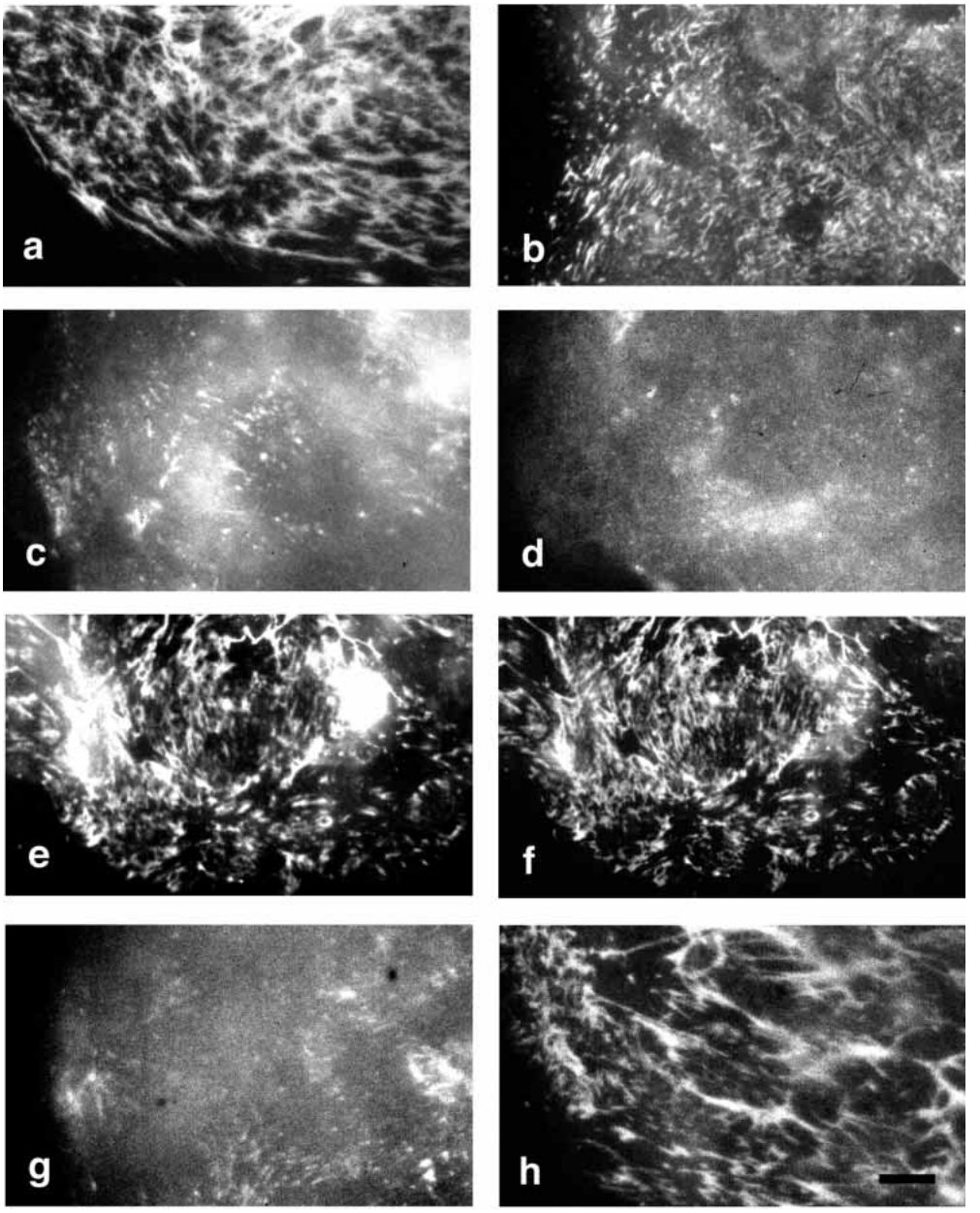

Fig. 1. Analysis of FN deposition by FRT-β1A and FRT-β1B cells in different culture conditions. Subconfluent FRT-β1A cells (a,b) and FRT-β1B cells (c,d) were cultured for 18 hours in 5% FBS (a,c) or 0% FBS (b,d). FN was detected by immunofluorescence with an antiserum to rat FN. (e,f) FRT-β1A cells were cultured for 18 hours in the presence of 4 μg biotinylated FN. FN fibrils were detected with an antiserum to rat FN (e) and biotinylated FN was detected with fluorescein-tagged avidin (f). (g,h) FRT-β1A cells were cultured for 18 hours in complete medium with (g) or without (h) the addition of the α5β1 FN receptor blocking antibody PB1 (1:100 dilution). The experiments were performed at least three times. Bar, 25 μm.

plaques, and assemble very few FN fibrils with respect to wild-type FRT cells (Cali et al., 1998). We speculated that FRT-β1B cells might be defective in one or more integrin-mediated signaling event(s) that might activate RhoA, and that lack of RhoA activity could be responsible for FRT-β1B cell phenotype. To prove this, FRT-β1B cells were transiently transfected with a vector carrying the constitutively activated form of RhoA, RhoAV14, fused to a myc tag. After 24 hours cells were fixed and double-stained for immunofluorescence detection with the anti-myc antibody (Fig. 4a,c,e) and with rhodamine-conjugated phalloidin (Fig. 4b), anti-paxillin antibody (Fig. 4d) or anti-FN antibody (Fig. 4f). The ability to assemble actin microfilaments (Fig. 4b), adhesion plaques (Fig. 4d) and FN fibrils (Fig. 4f) was rescued in a high number of transfected cells. The cells expressing RhoAV14 that organized FN fibrils and actin filaments were 83±7%, and 24±10% in mock transfected cells. Some of the myc-positive cells, although expressing microfilaments, had a round shape and lacked FN fibrils.

Treatment of FRT-β1B cells with CNF1 activates RhoA and induces the organization of fibrillar FN

In order to increase the amount of active RhoA in FRT cells expressing the β1B integrin, we treated the cells with CNF1, a toxin produced by pathogenic strains of *E. coli* that has been shown to directly bind and activate Rho (Fiorentini et al., 1997; Flatau et al., 1997; Schmidt et al., 1997). CNF1 has been shown to induce an increase in the content of actin stress fibers and focal contacts in cultured cells (Lacerda et al., 1997; Oswald et al., 1994). The activation of RhoA by CNF1 is correlated with a decrease in its electrophoretic mobility (Oswald et al., 1994). To determine if CNF1 was indeed causing a shift in the electrophoretic mobility of RhoA protein, FRT and FRT-β1B cells were treated with CNF1 and cell extracts were analyzed

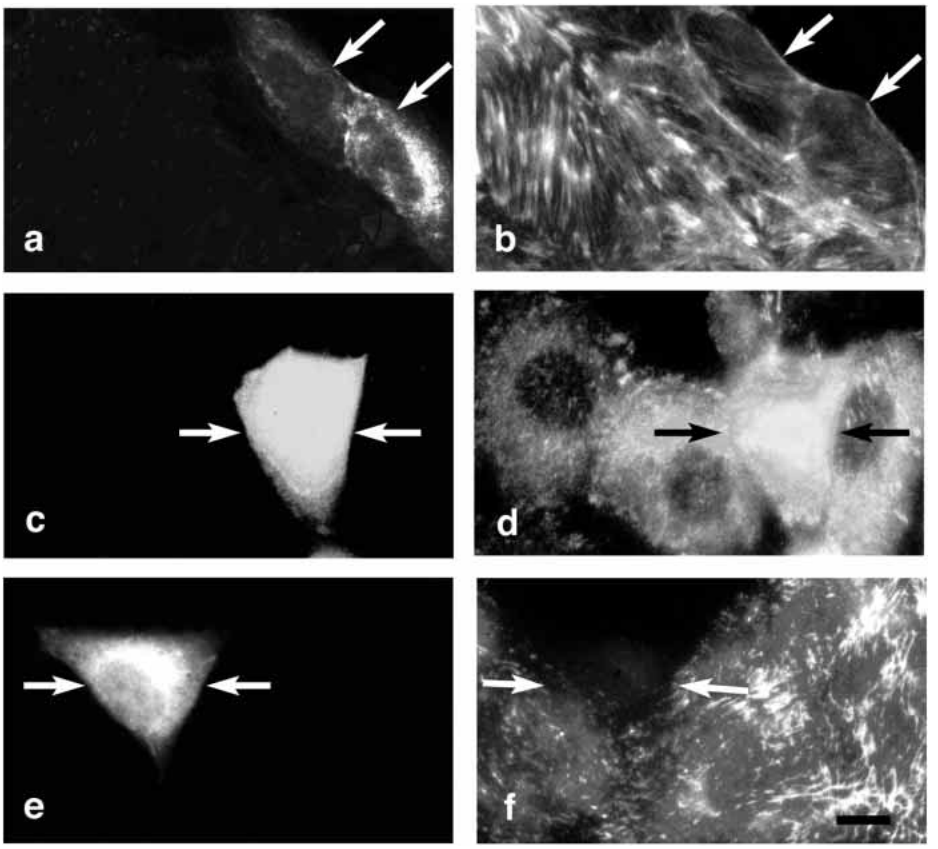

Fig. 2. Effect of dominant-negative RhoAN19 on FN assembly in FRT-β1A cells. FRT-β1A cells grown on glass coverslips were transfected with 2 μg pEXVmyctag RhoAN19 (see Materials and methods). Cells were stained and detected by immunofluorescence using the anti-myc antibody diluted 1:50 (a,c,e) to detect RhoAN19 expression and double stained with rhodamine-conjugated phalloidin (b), with anti-paxillin antibody diluted 1:100 (d) or with anti-FN antibody diluted 1:100 (f). Cells that were stained by the anti-myc antibody and show a reduction in actin microfilaments (a,b), adhesion plaques (c,d) and FN fibrils (e,f) are indicated by arrows. The experiments were performed at least five times. Bar, 10 μm.

by western blot using antibodies against RhoA. A reduction in the migratory pattern of RhoA in CNF1-treated cells was observed (Fig. 5). To determine if CNF1 was promoting FN assembly, FRT-β1B cells were grown in standard culture conditions for 48 hours, treated with CNF1 for 18 hours and examined by immunofluorescence using rhodamine-conjugated phalloidin, anti-paxillin antibody or anti-FN antibody. A great increase in actin polymerization (Fig. 6a-b), adhesion plaque organization (Fig. 6c-d) and FN assembly (Fig. 6e-f) was observed. These effects were already manifested by 6-8 hours of incubation, while changes in cell morphology due to CNF1 toxicity started to be seen after 24 hours. Similar results were observed when wild-type FRT cells were treated with CNF1 (data not shown).

TGF-β promotes the coordinated organization of FN matrix, actin microfilaments and adhesion plaques

TGF-β is a multifunctional cytokine capable of inducing a great number of effects, depending on the cell type (Clark et al., 1998). In thyroid cells TGF-β controls cell proliferation,

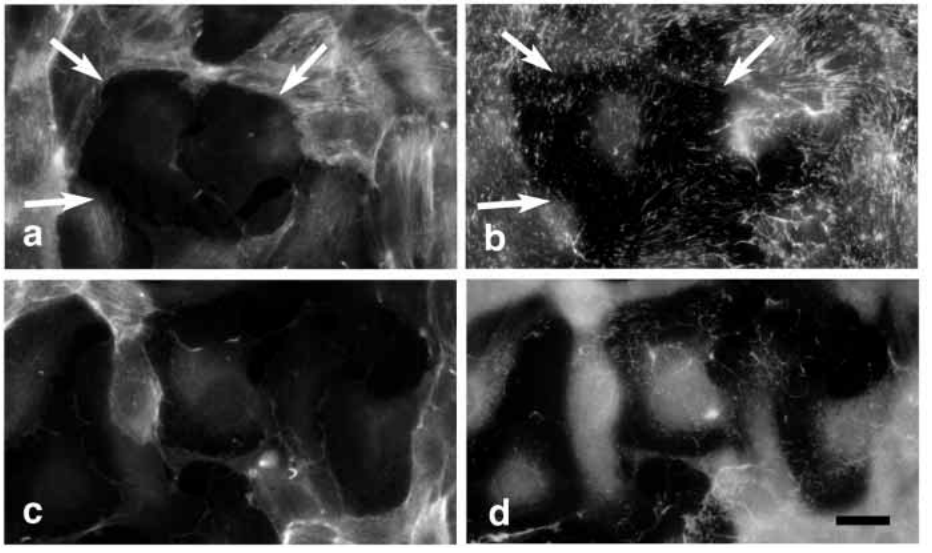

Fig. 3. Treatment of FRT-β1A cells with C3 transferase inhibits FN fibril formation. FRT-β1A cells were cultured on glass coverslips for 48 hours and treated with 1.5 μg/ml C3 transferase for 8 hours (a,b) or 18 hours (c,d). Cells were double-stained with rhodamine-conjugated phalloidin (a,c) and with the anti-FN antibody (b,d). Lack of microfilaments (a) and a reduction in FN fibrils (b) is observed in localized areas (arrows) at 8 hours. An almost complete loss of microfilaments (c) and of FN fibrils (d) is observed at 18 hours. The experiments were performed at least three times. Bar, 15 μm.

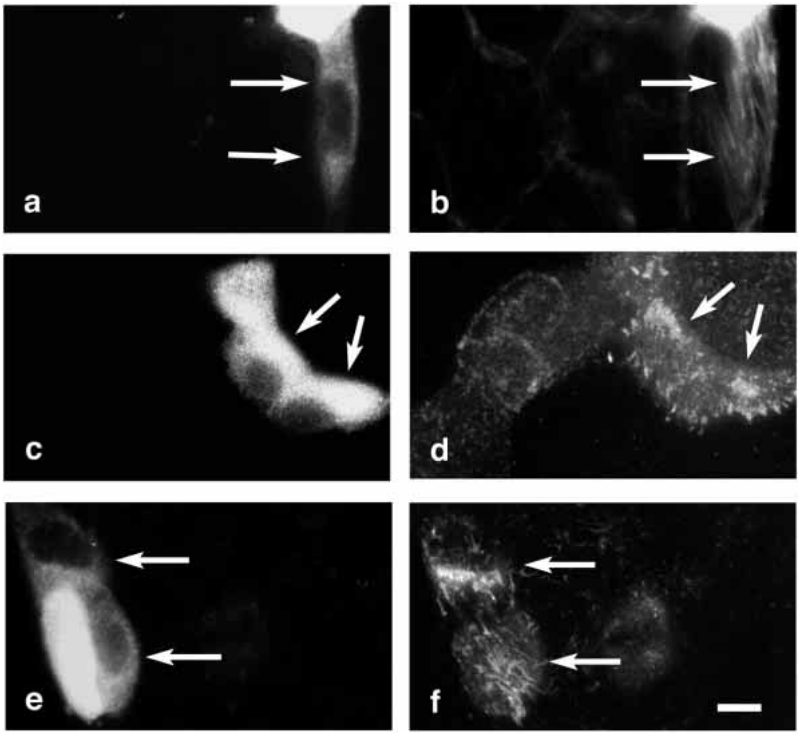

Fig. 4. Effect of the constitutively active form of RhoA (RhoAV14) on FN fibril assembly in FRT-β1B cells. FRT-β1B cells grown on glass coverslips were transfected with 2 μg pEXVmyctag V14Rho (see Materials and methods). Cells were stained for immunofluorescence detection using the anti-myc antibody (a,c,e) to detect RhoAV14 expression and double-stained with rhodamine-conjugated phalloidin (b), with anti-paxillin antibody (d) or with anti-FN antibody (f). Cells that are stained by the anti-myc antibody and show an increase in actin microfilaments (a,b), adhesion plaques (c,d) and FN fibrils (e,f) are indicated by arrows. Note that in all panels the fields are almost entirely filled with cells. The experiments were performed at least five times. Bar, 20 μm.

modulates some differentiated functions and acts as a potent inducer of stress fiber formation (Garbi et al., 1990). The exact mechanism by which this molecule exerts its intracellular effects is not known, though a recent report indicates that Rho activity might be required (Atfi et al., 1997). We treated FRT-β1B cells with 5 ng/ml of TGF-β. Cells were fixed and stained with rhodamine-conjugated phalloidin, anti-phosphotyrosine or anti-FN antibodies. TGF-β treatment induced within 24 hours the formation of a rich FN extracellular matrix paralleled by the formation of a great number of microfilaments and adhesion plaques (Fig. 7). However, at variance to what observed with CNF1, longer periods of incubation were needed to see these effects.

DISCUSSION

We report in this paper the involvement of RhoA in the process of FN fibrillogenesis in FRT thyroid epithelial cells. These cells are able to synthesize and secrete FN (Canipari et al., 1992) and to organize it into an insoluble fibrillar matrix with a polarized distribution. Unlike fibroblasts, where FN matrix is also present on the cell free surface (Wennerberg et al., 1996), FRT cells deposit FN only underneath the cells, at cell-substrate contact sites (Cali et al., 1998). We have generated two FRT-derived cell lines that constitutively express the human β1A or β1B integrin subunit and have demonstrated that β1 integrins are involved in FN assembly (Cali et al., 1998). We observed that both the exogenous FN supplied with the serum and the cellular newly synthesized FN can be incorporated into the same fibrils, indicating that they are recognized by the cells with similar efficiency. Thus the different ability to organize FN matrix observed in FRT-β1A and FRT-β1B cells is likely not due to differences in FN synthetic potential.

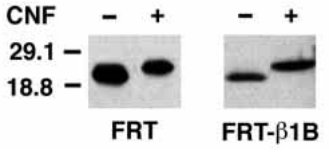

Fig. 5. CNF1 treatment induces a shift in the electrophoretic mobility of RhoA. Subconfluent FRT and FRT-β1B cells were treated for 3 hours with 0.2 ng/ml CNF1. Soluble proteins were separated by SDS/6-15%PAGE and analyzed by western blot using the anti-RhoA mAb diluted 1:1000. Molecular masses are indicated on the left side of the figure.

Using the specific function-blocking antibody PB1 we were able to demonstrate that the α5β1 dimers are essential for the process of FN fibrillogenesis. Since FRT cells are capable of sorting and retaining the β1 integrin receptors almost exclusively on the basolateral surface (Cali et al., 1998), it is conceivable that polarized distribution of α5β1 is responsible for the polarized assembly of FN. PB1 inhibition also suggests that α5β1 cannot be substituted by other integrins in the FN assembly process, although FRT cells express the αvβ3 integrin that can replace α5β1 in other cell model systems (Wennerberg et al., 1996).

To investigate the role of RhoA in FN assembly we have transiently expressed the dominant-negative RhoAN19 in FRT-β1A cells. A significant reduction in the amount of FN matrix assembly, paralleled by loss of microfilaments and reduction of focal adhesions, was observed in RhoAN19-expressing cells. A similar inhibition of FN assembly was also obtained upon exposure of FRT-β1A cells to the C3 transferase toxin. In quiescent Swiss 3T3 cells C3 transferase treatment also

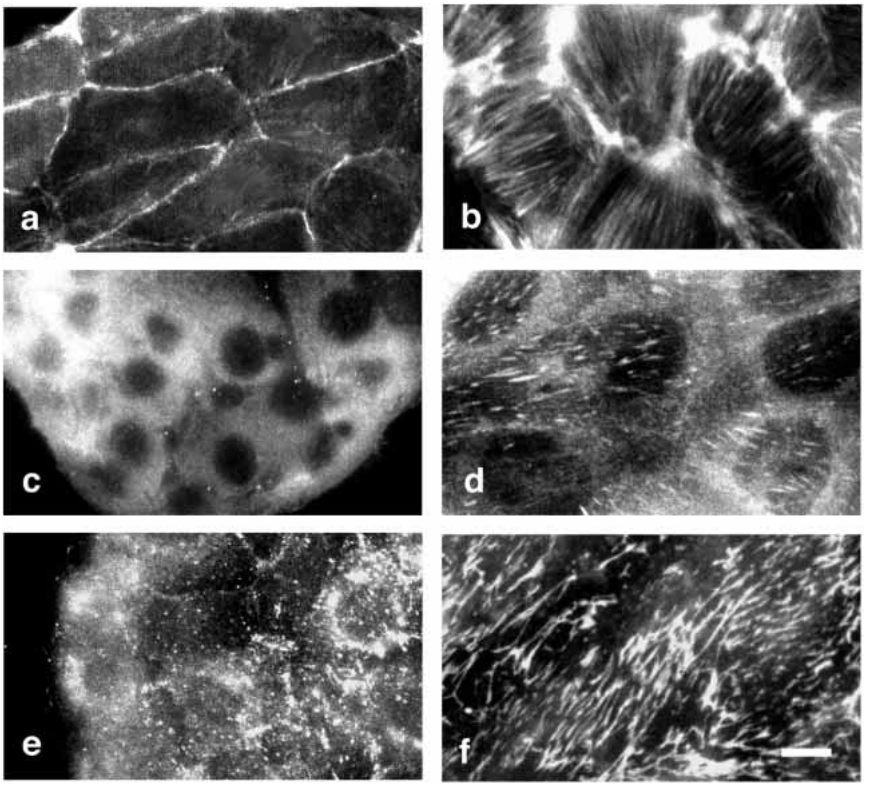

Fig. 6. Treatment of FRT-β1B cells with CNF1 promotes FN fibril assembly. FRT-β1B cells were cultured on glass coverslips for 48 hours and treated with 0.2 ng/ml CNF1 for 8 hours. Untreated cells (a,c,e) and CNF1-treated cells (b,d,f) were stained with rhodamine-conjugated phalloidin (a,b), with anti-paxillin antibody (c,d) and with anti-FN antibody (e,f). An increase in actin filaments (b), adhesion plaques (d) and FN fibrils (f) is evident. The experiments were performed at least three times. Bar, 15 μm.

determines a decrease in FN assembly (Zhong et al., 1998). In FRT-β1A cells, however, C3 transferase inhibited the formation of new fibrils and caused the removal of the FN matrix already deposited. Extracellular FN assembly should possibly be regarded as a dynamic process in which there is a continuous balance between FN fibril formation and degradation. RhoA activity might be a permanent requirement in the interplay between the intracellular actin cytoskeleton and the extracellular FN matrix that is needed in these processes.

A major finding of this report is the demonstration that FN

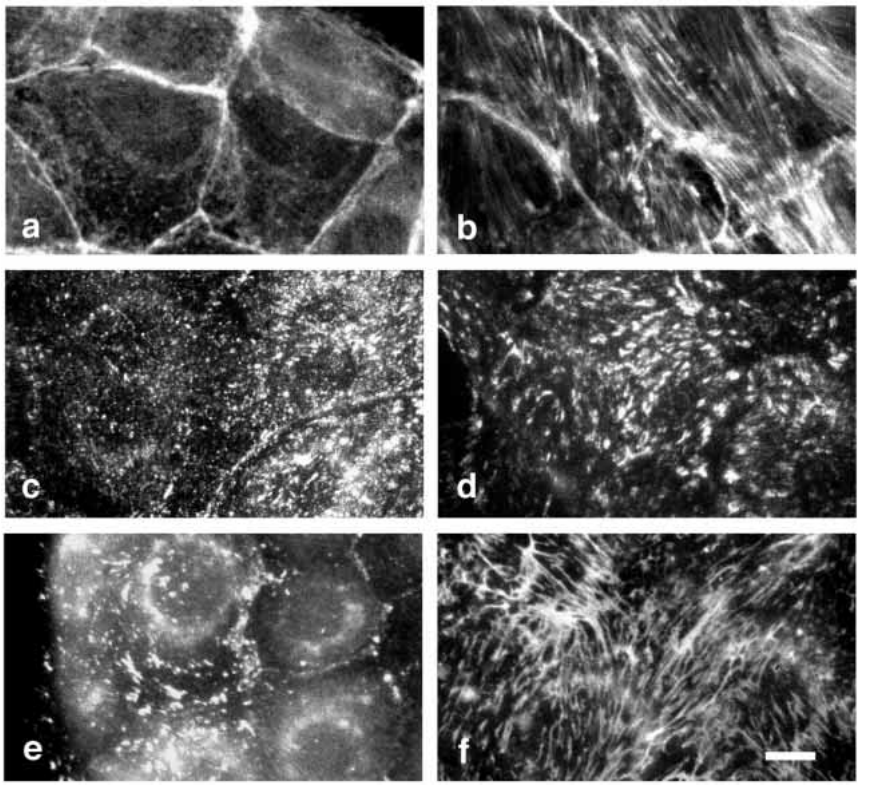

Fig. 7. Treatment of FRT-β1B cells with TGF-β induces FN matrix organization. FRT-β1B cells were cultured on glass coverslips for 48 hours and treated with 5 ng/ml TGF-β for 24 hours. Untreated cells (a,c,e) and TGF-β treated cells (b,d,f) were stained with rhodamine-conjugated phalloidin (a,b), with anti-phosphotyrosine antibody (c,d) and with anti-FN antibody (e,f). A prominent increase in actin filaments (b), adhesion plaques (d) and FN fibrils (f) is observed. The experiments were performed at least three times. Bar, 10 μm.

fibrillogenesis can be restored in FRT-β1B cells by transient expression of the constitutively activated RhoAV14 or by treatment with CNF1. In both experiments microfilament assembly and adhesion plaques were concomitantly stimulated. These results indicate that β1B inhibition is not permanent and that it can be overcome. Moreover they suggest that in FRT-β1B cells the signal transduction pathway downstream of RhoA operates as in wild-type FRT cells and that β1B expression affects some other step of the integrin signaling pathway. FRT-β1B cells do not organize microfilaments and have a reduced number of very small adhesion plaques. These phenotypic properties correspond to what is observed in fibroblasts where RhoA activity is inhibited by the expression of RhoAN19 (Clark et al., 1998). It is therefore conceivable that FRT-β1B cells have impairment in the process that leads to RhoA activation.

The β1B human integrin is an alternatively spliced variant of the β1 subunit, where the cytosolic tail of the molecule is shorter than in β1A and carries a specific COOH-terminal sequence 12 amino acids long (Altruda et al., 1990; Balzac et al., 1994). It has been recently demonstrated that β1B expression in GD25 β1 null cells interferes with the αvβ3-dependent FN matrix assembly and that β1B is present at the cell surface in an inactive conformation (Retta et al., 1998). It has been proposed that β1B could bind and sequester molecules necessary for integrin signal transduction. A similar mechanism could be acting in FRT cells, although other possibilities, such as association to α subunits, cannot be excluded. To date no direct interactions between integrins and RhoA have been reported. It has been proposed that in LOX melanoma cells the α6β1 integrin signaling regulates the tyrosine phosphorylation state of p190 GAP and therefore influences the downstream signaling pathway through Rho (Nakahara et al., 1998).

How does the constitutive activation of RhoA promote FN assembly? Since RhoA is able to induce integrin clustering (Hotchin and Hall, 1995; Machesky and Hall, 1997), it is plausible that FN assembly can occur by recruitment of the endogenous α5β1 integrins. An increase in FN assembly also occurs in CNF1-treated wild-type FRT cells. RhoA could also determine the acquisition of a high-affinity state of the β1B integrin subunit. However, only small variations in the state of integrin activation have been observed as a consequence of RhoA activity changes (Zhong et al., 1998). A critical role in FN fibrillogenesis appears to be played by Rho-mediated cell contractility, which could generate tension in the FN molecules and expose cryptic self-assembly sites (Zhong et al., 1998).

The family of transforming growth factors beta has profound regulatory effects on cell growth and differentiation (Clark et al., 1998; Roberts et al., 1990). TGF-β modulates the synthesis and accumulation of ECM components and the expression of cell surface receptors for ECM components (Ignotz and Massague, 1987). In many cell types TGF-β promotes actin cytoskeleton organization. We have shown that, in FRTL5 rat thyroid epithelial cells, TGF-β induces stress fiber formation and changes in the extracellular matrix organization (Garbi et al., 1990). Although the mechanism of receptor activation is reasonably understood, there is little information about the potential downstream targets of the receptor complex. Recent observations suggest that Rac (Mucsi et al., 1996) or Rho-like GTPases (Atfi et al., 1997) mediate some effects of TGF-β signaling. Since TGF-β effects on cytoskeleton and ECM mimic Rho activation, we speculated that there might be a possible link between TGF-β activity and Rho in the FRT cell model system. Indeed, TGF-β promoted assembly of FN and organization of microfilaments in FRT-β1B cells to the same extent as CNF1 induced RhoA activation.

In conclusion, RhoA activity is needed to determine α5β1-dependent FN assembly in FRT epithelial cells. β1B possibly inhibits FN assembly by interfering with some step of the integrin signal transduction pathway.

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